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<b>(54) Title:</b> CAROTENOID AGENT FOR INHIBITING THE CONVERSION OF EPITHELIAL CELLS TO TUMOURS <b>(57) Abstract</b> <p>The invention is a carotenoid agent for inhibiting the conversion of epithelial cells to tumours, the agent including an effective amount of a water insoluble carotenoid component in a suitable non-toxic carrier medium.</p>		

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## CAROTENOID AGENT FOR INHIBITING THE CONVERSION OF EPITHELIAL CELLS TO TUMOURS

### Field of the Invention

5 The invention relates to a carotenoid agent for inhibiting the conversion of epithelial cells to tumours and a method for such treatment. More particularly, the invention relates to a carotenoid agent for inhibiting the conversion of melanocytes to melanomas and a method for such treatment.

### Background to the Invention

10 Although the description which follows is specifically directed to melanocytes and melanomas, it will be clear to a person skilled in the art that similar comments apply with respect to other types of epithelial cells.

Melanomas originate from a change in normal skin cells, melanocytes, which produce the brown pigment melanin we recognise as tan. Moles and freckles result from areas of the skin with many melanocytes.

15 Melanomas are caused generally by the exposure of skin to sunlight. Persons of fair complexion have the greatest risk especially those who develop moles.

The influence of light on melanocytes is one way by which they can be changed to grow and divide differently, possibly causing a melanoma. The melanomas may be malignant, spreading to other parts of the body. Melanomas which do not spread are called benign melanomas.

20 Although melanomas normally form on exposed skin they can start in places such as the mouth.

Melanomas grow in size and need to be surgically removed before they spread and invade other parts of the body. If the melanomas spread to the inner organs, removal and treatment is more difficult and chemotherapy or radiotherapy need to be employed. For this reason, if melanocytes can be inhibited from converting to melanomas, the associated problems with the treatment of melanomas can be similarly reduced.

25 It has been hypothesised that carotenoids and in particular beta-carotene may reduce the risk of breast, lung, colon, prostate and cervical cancer, heart disease and stroke and may retard macular degeneration. In this respect, one hypothesis is that in mammals beta-carotene is converted to vitamin A and vitamin A analogues or retinoids (see Moon RC: Comparative aspects of carotenoids and retinoids as chemopreventive agents for cancer. J Nutr 119:127-134, 1989). It is this pro-vitamin A activity and the ability to prevent oxidative damage that has made carotenoids and, in particular, beta-carotene a

compound of interest in chemopreventive studies. For instance, anti-oxidants are used, amongst other things, to quench free radicals that are by-products of normal metabolism in cells.

Beta-carotene has also been used in the treatment of erythropoietic protoporphyria (EPP).

- 5 EPP is a genetic disease causing an inadequacy in the metabolism of porphyrin compounds. It results in a rapid blistering of the skin on exposure to sunlight.

Although, the effectiveness of carotenoids has been hypothesised in reducing the risk of certain instances of cancer, no studies have demonstrated the possible inhibiting effect of carotenoids in the conversion of melanocytes to melanomas.

- 10 For this reason, investigations were undertaken to determine the efficacy of carotenoids in inhibiting or reducing the conversion of melanocytes to melanomas. As part of this investigation, it was necessary to consider the difficulties in the use of carotenoids in human applications because of the nature and chemical properties of carotenoids.

- 15 Carotenoids are lipophilic and therefore not soluble in water in useful quantities. It is believed that they are transported in the bloodstream in conjunction with low density lipoproteins.

- To date several in vitro studies have taken place to determine the effect of beta-carotene on normal and transformed cell types using solvents to solubilise the beta-carotene such as tetrahydrofuran, butanol, chloroform, hexane, dimethylsulfoxide, ethanol or in a liposome  
20 micelle. Previous liposome preparations have shown toxicity in cell line cultures as well as being limited in application (see Bertram JS, Pung A, Churley M, et al: Diverse carotenoids protect against chemically induced neoplastic transformation. *Carcinogenesis* 12:671-678, 1991; Hazuka MB, Prasad-Edwards J, Newman F, et al: Beta-carotene induces morphological differentiation and decreases adenylate cyclase activity in  
25 melanocyte cells in culture. *J Am Coll Nutr* 9:143-149, 1990; Schultz TD, Chew BP, Seaman WR, et al: Inhibitory effect of conjugated dienoic derivatives of linoleic acid and beta-carotene on the in vitro growth of human cancer cells. *Canc Letters* 63:125-133, 1992; Schwartz JL, Shklar G: The selective cytotoxic effect of carotenoids and a-tocopherol on human cancer cell lines in vitro. *J Oral Maxillofac Surg* 50:367-373, 1992;  
30 Schwartz JL, Tanaka J, Khandekar V, et al: Beta-Carotene and/or Vitamin E as modulators of alkylating agents in SCC-25 human squamous carcinoma cells. *Canc Chemother Pharmacol* 29:207-213, 1992; Zhang L-X, Cooney RV, Bertram JS: Carotenoids enhance gap junctional communication and inhibit lipid peroxidation in C3H/10T1/2 cells: relationship to their cancer chemopreventive action. *Carcinogenesis* 12:2109-2114, 1991;  
35 and Zhang L-X, Cooney RV, Bertram JS: Carotenoids up-regulate connexin 43 gene expression independent of their provitamin A or antioxidant properties. *Canc Res*

52:5707-5712, 1992). These solvents have been found to have a toxic effect which is dose dependent. These solvents are also incompatible with human blood or lymph for the purposes of intravenous or injectable preparations.

Thus, in administering carotenoids, care must be taken to use appropriate carriers.

- 5 Accordingly we carried out in vitro studies to determine the efficacy of carotenoids in inhibiting the conversion of epithelial cells to tumours and more particularly, the conversion of melanocytes to melanomas.

### Summary of the Invention

- 10 According to the present invention, a carotenoid agent is provided for inhibiting the conversion of epithelial cells to tumours, the agent including an effective amount of a water insoluble carotenoid component in a suitable non-toxic carrier medium.

The invention also provides a method of inhibiting the conversion of epithelial cells to tumours, including the step of applying to the cells, an effective amount of a water insoluble carotenoid component in a suitable non-toxic carrier medium.

- 15 The invention further provides a method of treating epithelial cells, so as to inhibit their conversion into tumours, including the step of applying to the cells, an effective amount of a water insoluble carotenoid component in a suitable non-toxic carrier medium.

- 20 The epithelial cell may be of mammalian origin. Preferably, the cell is a skin cell. It is particularly preferred that the skin cell is a melanocyte. In an even more preferred form of the invention, the epithelial cell is a human cell, and in a particularly preferred form of the invention, a human melanocyte.

- 25 Preferably, the water insoluble carotenoid component includes beta-carotene. In a yet further preferred form of the invention, the water insoluble carotenoid component includes from 2% to 50% by weight of beta-carotene. It is further preferred that the water insoluble carotenoid component includes from 20% to 40% by weight of beta-carotene. Even more preferably, the water insoluble carotenoid component includes 30% by weight of beta-carotene.

- 30 Preferably, the carrier medium includes a suspending agent selected from the group comprising fatty acids, triglyceride lipids, non-saponifiable lipid preparations, soluble hydrocarbons and combinations thereof.

Preferably, the triglyceride lipids are selected from the group comprising fats and/or oils derived from plant sources. The fats and/or oils may be derived from plant sources (in which case, it is particularly preferred that seed oils, such as cotton seed oil, sunflower oil or combinations thereof are used), or from animal sources (such as meat and fish). Seed oils are particularly preferred, and soya bean oil is an especially preferred oil for use in the carrier medium.

Preferably further, the water insoluble carotenoid component constitutes from 0.1 % to 10% by weight of the agent. It is particularly preferred that the water insoluble carotenoid component constitutes from 1 % to 5% by weight of the agent.

10 The non-toxic carrier medium preferably also includes an emulsifier. Preferably, the emulsifier is selected from the group including Tweens, glycerol fatty acid esters and acetylated esters of fatty acids. Glycerol mono-oleate is a particularly preferred emulsifier.

Preferably, the non-toxic carrier medium also includes a water soluble dispersing agent. It is particularly preferred that the water soluble dispersing agent is a sugar or a polyol. It is especially preferred that the water soluble dispersing agent is selected from the group including sorbitol and glycerol.

In all forms of the invention, preferably, the effective amount of the carotenoid agent contacting the melanocyte cells is from 0.1 to 10.0 micrograms/ml and more preferably, 0.3 to 3.0 micrograms/ml. These concentrations are achieved by dilution with suitable diluents. More preferably the diluting solution is selected from media suitable for the growth of the cells, aqueous buffers, normal intravenous preparations (including isotonic saline or 5% dextrose solution) and blood serum and combinations of the foregoing.

The term "mixture" as used herein is intended to include various physical forms including emulsions, solutions and crystal suspensions.

## 25 Examples

The following examples demonstrate the effectiveness of carotenoid compositions in the treatment of melanocytes and the relative non-toxicity of those compositions.

The examples that follow will now be described with reference to the accompanying drawings, in which:

30 Figure 1 is a graph showing the effect of beta-carotene on DNA synthesis in normal human melanocytes. In summary, while DNA synthesis was largely inhibited at the highest beta-carotene dose, the lower doses had little or no effect. The 1.0 ug/ml dose was chosen as the pre-treatment dose for the UV experiments as it offered the highest beta-carotene concentration achievable with minimal effect on DNA synthesis. Beta-carotene was

incubated with the melanocytes for 24 hours. Each data point is the mean of 6 wells +/- % std. error as compared to control.

Figure 2 is a graph showing the viability of normal human melanocytes following UV-B irradiation with and without beta-carotene. In summary, immediately following 500 mJ/cm<sup>2</sup> of UV-B (0.05) hr, cell viability increased almost 20% with beta-carotene present as compared to UV-B alone. This effect remained for the duration of the time course. Each data point is the mean of 3 wells +/- % std. error as compared to controls.

Figure 3 are graphs showing the northern analysis of melanocytes exposed to UV-B irradiation with and without beta-carotene. In summary, the largest induction of c-jun by UV-B occurred 24 hours following a 500 mJ/cm<sup>2</sup> exposure (36.2 fold over basal level). Maximal induction of c-fos occurred 2 hours following irradiation with expression level 57 fold above basal. The induction of c-fos by UV-B was essentially blocked by beta-carotene. C-jun expression was increased 11.1 fold by beta-carotene four hours following irradiation while at 24 hours, the induction by UV-B was completely blocked. Basal expression level of these genes without beta-carotene or UV-B has been subtracted from all RNA levels shown. Blots were corrected for loading by 18S.

Figure 4 is a graph showing DNA synthesis in normal human melanocytes with and without beta-carotene. In summary, beta-carotene pre-treatment increased DNA synthesis 24 hours following 400 mJ/cm<sup>2</sup> of UV-B. This effect was not seen 4 hours after irradiation. Each data point is the mean of 6 wells +/- % std. error as compared to controls.

Figure 5 is a graph showing the spectrum distribution for the UVP bulbs used in the Stratalinker Crosslinker apparatus which was used in the experiments.

Figure 6 shows four histograms depicting the results of experiments comparing the effects of beta-carotene treatment on melanocytes exposed to UV-B irradiation. In summary, melanocytes were treated for 24 hours with or without beta-carotene (1 ug/ml) with the appropriate controls. Media was then removed and cells were exposed to 100 or 500 mJ/cm<sup>2</sup> of UV-B. Fresh media was then added and cells were incubated for an additional 1, 4 and 24 hours and total RNA isolated. This histogram is representative of four independent Northern blots (corrected for loading) from four normal Caucasian melanocyte lines.

Figure 7 shows the results of beta-carotene treatment on the DNA binding activity of cells exposed to 100 mJ/cm<sup>2</sup> UV-B irradiation.

Figure 8 shows the results of beta-carotene treatment on the DNA binding activity of cells exposed to 500 mJ/cm<sup>2</sup> UV-B irradiation. In summary, gel mobility shift assay using a NF Kappa B consensus sequence was performed to determine DNA binding activity of nuclear

extracts from four normal Caucasian melanocyte (NCM) lines. NCM were pre-treated with 1.0 ug/ml of beta-carotene for 24 hours followed by 500 mJ/cm<sup>2</sup> of UV-B and assayed at 1, 4 and 24 hours following irradiation.

5 Figure 9 shows the effects of treating human melanocytes exposed to O-Tetradecanoylphorbol 13-acetate (TPA), 3-Isobutyl 1-Methyl Xanthine (IBMX) and a combination of TPA and IBMX on the total RNA synthesis of those melanocytes subsequently irradiated with UVB. In summary, melanocytes were treated for 24 hours with or without TPA (10 ng/ml) or IBMX (0.1 mM) with the appropriate controls. Media was then removed and cells exposed to UV-B received a dose of 100 mJ/cm<sup>2</sup>. Fresh media  
10 was then added with or without TPA or IBMX and cells were incubated for 1 hour following irradiation and total RNA was isolated. This histogram is representative of two independent Northern blots (corrected for loading) from two normal neonatal Caucasian melanocyte lines.

15 Figure 10 shows an overview of the intracellular mechanisms putatively responsible for the reaction of human melanocytes to ultra-violet radiation exposure.

Figures 1 and 5 refer to "Percent Tritiated Thymidine Incorporation" which is a measure of DNA synthesis activity.

Details of the experiments conducted in relation to the invention are as follows.

## Materials

### 20 (a) Cell Cultures

Human neonatal foreskins were placed in 0.25% trypsin at 4°C overnight. Following this incubation, the tissue was scraped to recover the melanocytes. The melanocytes were cultured in MCDB 151 (Sigma) medium containing 2% fetal calf serum, 0.3% bovine pituitary extract (Clonetics Corp.), 10 nanograms/ml TPA, 2 mM CaCl<sub>2</sub>, 5 micrograms/ml  
25 insulin and 0.1 mM IBMX (Sigma).

### (b) Chemicals

30 The beta-carotene was isolated from the alga *Dunaliella salina* and represented 85-90% of the total carotenoids, with half of the balance consisting of oxycarotenoids (lutein and zeaxanthin) and the remaining half of alpha-carotene. Gamma-carotene is normally undetectable as characterised by high pressure liquid chromatography. The soya bean oil was isolated from soya beans. A crystalline suspension of beta-carotene and soya bean oil was created. This resultant phase was then emulsified into the composition described. It was then sterilised by heat or filtration. Prior to testing on the cell lines each vial of the



composition was sub-aliquoted into cryogenic vials (Costar) with a fresh vial used for each experiment. Throughout all procedures, beta-carotene was protected from direct light.

The details of the emulsified beta-carotene composition are as follows:

		% by weight
5	(i) Beta-carotene	2.4%
	and	
	(ii) SOY being:	
	Soya bean oil	6.8%
	Glyceryl mono-oleate	7.2%
10	Glycerol	66.7%
	Water	16.9%

This composition can be prepared by the following method. A crystalline suspension of beta-carotene in soya bean oil is heated and glyceryl mono-oleate is added. This oil phase is dispersed in the glycerol-water phase by high shear mixing followed by homogenisation at 60-70°C. Typically, a homogenisation pressure of 8,000 to 10,000 PSI is used, however, this pressure will vary according to the machine that is used. The resulting product is then sterilized by heat processing. Typically, heat processing is effected by autoclaving at 121°C for 15 minutes in a pack for dispensing (3ml glass vial). Optionally, 0.3% of the anti-oxidant tocopherols is added to overcome any toxicity that may develop over a period of time.

### (c) Experimental Conditions

Ultra-violet ("UV") radiation can cause severe damage to cells, including genetic mutations, the promotion of cancer, and other adverse conditions. The potentially harmful effects of UV radiation for humans are mainly due to the UVB spectrum (ie, radiation having a wavelength of between 290 and 320 nanometres) (See: Gilchrest BA, Soler NA, Staff JS and Mihm MC Jr: "The human sunburn reaction: histologic and biochemical studies" J. Am. Acad. Dermatol 5: 411-422 (1981)). Another form of UV radiation is called "UVC". UVC radiation in sunlight typically has a wavelength of the order of 254 nanometres. It is not as biologically relevant as UVB radiation however, because the UVC radiation in sunlight is strongly absorbed by the earth's ozone layer. Accordingly, in the present study, we investigated the effects of UVB irradiation by melanocytes, and the changes to those effects by treating those cells with carotenoids. UVC wavelengths were blocked out of the experiments as the cells were incubated in plastic vessels.

UVB irradiation of the melanocytes was performed with a Stratalinker Crosslinker (Stratagene) equipped with five UVP bulbs which exhibit an emission maximum centred at 302 nm as measured by a UVX-31 radiometer (UVP Inc.). Wavelengths shorter than 280 nm were cut off by keeping lids on tissue culture dishes. Figure 5 shows the spectrum distribution for the Stratalinker's UVP bulbs.

**(d) RNA analysis**

Total RNA was isolated by detergent lysis followed by phenol-chloroform extraction and ethanol precipitation. 15 micrograms of RNA was size-fractionated on denaturing formaldehyde/agarose gels and transferred to nylon filters by capillary blotting. Blots were exposed to  $^{32}\text{P}$ -labelled cDNA probes and hybridized at 42°C for 24 hours in 50% formamide, 2X SSC, 5X Denhardts, 0.1% SDS, 10% dextran sulfate and 100 micrograms/ml salmon sperm DNA. After hybridization, filters were washed to a stringency of 0.5-0.1X SSC at 60°C and autoradiographed at -80°C for three to ten days using Fuji film. Autoradiographs are quantitated by densitometry using the GS-365 software (Hoefer Scientific).

**(e) Gene Probes**

The c-jun probe was a 1.2 kb Sal I/Hpa I fragment from the plasmid pHJ (Bohmann, 1987). The c-fos probe was a 1.8 kb Xho I/Eco R I fragment from the plasmid BK 28 (Verma, 1988). The 18S probe was isolated by an EcoRI digest and isolation of the 5.6 kb fragment from the plasmid pB (Gonzalez, 1988).

**(f) Cell counting and DNA synthesis measurements**

Cells were harvested with 0.25% trypsin and counted on a Coulter Counter (Coulter Instruments). Viability was determined by trypan blue exclusion. DNA synthesis was measured by labelling with (methyl- $^3\text{H}$ )-thymidine (2.5 mCi/ml, 20Ci/mmol, Dupont-New England Nuclear) added to the medium during the last 4 hours of incubation. Melanocytes were harvested using a Ph.D. cell harvester (Cambridge Research Inc.). The amount of radioactivity incorporated was determined by liquid scintillation counting (LS5000TD, Beckman Instruments).

**(g) Nuclear protein extracts**

Cells were grown to approximately 50-60% confluency and treated as described in the experimental protocol described in section (i) below. The cells were allowed to swell in hypotonic buffer (10 mM HEPES, pH 7.8, 0.1mM EDTA, 10 mM KCC, 1mM DTT, PMSF for 15 minutes on ice). Cytosolic protein fraction was obtained after the addition of Nonidet P-40 to a final concentration of 0.5% and separation from the nuclei by centrifugation (500 x g, for 5 minutes). Nuclear proteins were extracted from the

remaining pellet by dialysis with hypertonic buffer (20  $\mu$ M HEPES, pH 7.9, 350 mM NaCl, 1Mm DTT, 1mM PMSF) for 2 hours at 10°C. Isolated nuclear protein was frozen and stored at -80°C. Quantitation was calculated from optical density readings of 230, 260 and 320 wavelengths.

#### 5 (h) Gel mobility shift assays

Five microgram samples of each extract were incubated with 10,000 cpm of a  $^{32}$ P-labelled consensus oligonucleotide of NF Kappa B. Following a 1 hour incubation, samples were electrophoresed in a low ionic strength polyacrylamide gel. Quantitation of protein: DNA complexes was accomplished by densitometry.

#### 10 (i) Experimental Protocol

The following experimental timetable was adopted:

		UVB Irradiation		Assay Times	
		-48.....	-24.....	0.....	0.05.....1.....2.....4.....24
15	Melanocytes Plated	Beta Carotene 1 microgram/ml		Beta-carotene Removed, Irradiated, Media Without Beta-carotene Added	Post Irradiation

- 20 Normal human melanocytes were seeded into the appropriate vessel (6 well plate, 96 well plate, 75cm or 175 cm flask) and allowed to grow to 50-60% confluency. In a "treated" group, fresh media and 1.0 microgram/ml of beta-carotene were then added and the cells were incubated for 24 hours with the beta-carotene. After incubation, the cells were washed twice with PBS and exposed to 500 mJ/cm<sup>2</sup> of UV-B. Fresh media (without beta-carotene) was added following irradiation and cells were incubated for 1, 2, 4 and 24 additional hours. The 0.05 hour time indicates cells were harvested within 3 minutes following UV-B exposure. A "control" group received precisely the same treatment as the "treated" group, with the exception of being treated with beta-carotene.

#### Discussion of results

- 30 Figure 1 shows that while the DNA synthesis was largely inhibited at the highest beta-carotene dose, the lower doses had little or no effect. In this regard, the 1.0 microgram/ml was chosen as the pre treatment dose for the UV experiments as it offered the highest beta-carotene concentration achievable with minimal effect on DNA synthesis. Beta-carotene was incubated with the melanocytes for 24 hours. Each data point is the mean of 6 wells  
35 +/- % std. error as compared to control.

Figure 2 shows that immediately following 500 mJ/cm<sup>2</sup> of UV-B (0.05 hr), cell viability increased almost 20% with beta-carotene present as compared to UV-B alone. This effect remained for the duration of the time course. Each data point is the mean of 3 wells +/- % std. error as compared to controls.

- 5 Figure 3 shows the largest induction of c-jun by UVB occurred 24 hours following a 500 mJ/cm<sup>2</sup> exposure (36.2 fold over basal level). Maximal induction of c-fos occurred 2 hours following irradiation with expression level 57 fold above basal. The induction of c-fos by UVB was essentially blocked by beta-carotene. C-jun expression was increased 11.1 fold by beta-carotene four hours following irradiation while at 24 hours the induction  
10 by UVB was completely blocked. Basal expression level of these genes without beta-carotene or UVB has been subtracted from all RNA levels shown. Blots were corrected for loading by 18S.

Figure 4 shows that tritiated thymidine incorporation was increased 24 hours after irradiation in melanocytes treated with beta-carotene.

- 15 Without wishing to be limited to any specific theory, it appears that the mixture illustrated is a superfine emulsion.

Referring now to Figure 6, the histograms shown in that figure are representative of four independent Northern blots. On the left, are shown the results following 100 mJ/cm<sup>2</sup> UVB and on the right, the results following 500 mJ are shown. As shown in the histograms in Figure  
20 6, c-fos and c-jun RNA levels increased rapidly, with the highest expression level reached one hour after irradiation. This induction was transient, however, as expression returned to basal levels x 4 hours after irradiation. C-jun was at almost double the expression level at 1 hour following 500 mJ/cm<sup>2</sup>, when compared with 100 mJ/cm<sup>2</sup> irradiation. C-fos expression was also higher subsequent to irradiation with 500 mJ/cm<sup>2</sup>. Beta-carotene did  
25 not alter the UVB-induced C-fos expression and had only a slight effect on the C-jun.

Referring now to Figure 7, we then isolated nuclear protein from the melanocytes and looked at the DNA binding activities, subsequent to irradiation. The activity for the transcription factor NF Kappa B was essentially unaffected over this time course. Nuclear factor Kappa B (NF Kappa B) activation in the cytoplasm is known to be due to the  
30 dissociation of an inactive NF Kappa B-inhibitor of nuclear factor Kappa B complex. (See Simon, MM *et al*: "UVB light induces Nuclear Factor Kappa B (NF Kappa B) activity independently from chromosomal DNA damage in cell-free cytosolic extracts": The Journal of Investigative Dermatology: 102 (No 4), 422 (April 1994)). However beta-carotene slightly decreased NF Kappa B binding activity. Although the dose of 100  
35 mJ/cm<sup>2</sup> did not induce binding activity above basal level, beta-carotene did modify NF Kappa B activity.

Figure 8 shows the results of DNA binding activity of normal Caucasian melanocytes following irradiation with 500 mJ/cm<sup>2</sup> UVB. A representative autoradiograph of NF Kappa B activity is shown to the left and a histogram of this to the right. The lowest activity level is seen 24 hours following irradiation.

- 5 In Figure 7 beta-carotene was shown to have had a moderate effect on NF Kappa B at 100 mJ/cm<sup>2</sup>, while at the higher dose of 500 mJ/cm<sup>2</sup>, beta-carotene inhibited NF Kappa B activity by more than 50% in the first hour following irradiation. These data demonstrate that at the higher UVB dose, NF Kappa B binding activity is down regulated.

- 10 Figure 9 shows the results from Northern analyses of normal neo-natal Caucasian melanocytes cultured in TPA, as well as IBMX. We then looked at the effect of these media conditions on C-jun induction, subsequent to irradiation with 100 mJ/cm<sup>2</sup> of UVB. The melanocytes were treated with and without TPA and IBMX for 24 hours, then irradiated and incubated for an additional hour with and without the relevant factor. Compared to irradiated melanocytes in complete medium (ie medium which included both
- 15 TPA and IBMX), C-jun induction decreased approximately 50%. In addition, the irradiated melanocytes lacking TPA and IBMX simultaneously reduced C-jun expression to the levels seen in all of the non-irradiated conditions. RNA levels were unaffected by the absence of TPA, IBMX or their combination in melanocytes not exposed to in UVB. At this low dose of 100 mJ/cm<sup>2</sup>, it seems that melanocytes require both TPA and IBMX
- 20 for complete induction of C-jun.

### Conclusions

- The scientific literature shows that a number of transcription factors are likely to be involved in response to DNA damage by ultraviolet radiation. These include the C-fos and C-jun genes, as well as the NF Kappa B complex and the AP-1 complex. A schematic
- 25 overview of the somewhat complex putative mechanisms involved in mediating the response to ultraviolet radiation damage to the DNA of normal melanocytes is depicted in Figure 10. We conducted a series of experiments on cultured normal melanocytes (examined by Northern analysis for their expression of c-fos and c-jun, following exposure to ultraviolet-B (UVB) radiation). The data obtained from experiments which investigated
- 30 the effect on c-fos and c-jun genes have presented somewhat conflicting outcomes. The discrepancies observed may be due to a number of influencing factors, such as the possibility that the ultraviolet radiation doses used are not appropriate for studying the effects of such irradiation in the c-fos and c-jun genes in epithelial cells, or the effects of carotenoids on the response evoked. Accordingly, it may be necessary to study the effects
- 35 of irradiation and carotenoid treatments on these genes, under different experimental conditions. In addition, the possibility remains that other members of the jun and fos

family of genes (eg, jun-B, jun-D, fos-B, fra I, fra II) could play a more significant role in the response to ultraviolet irradiation on normal epithelial cells, and the effect of beta-carotene on that response. These unresolved questions will require further investigation.

5 On the other hand, we have recently been able to conduct experiments which investigated the more recently discovered properties of the NF Kappa B complex, and the results of those studies show a statistically significant result for beta-carotene protection against the UV- damage response mediated by that complex. While we do not express here any definitive and binding theory to account for these observations, it appears that the NF Kappa B complex is a more attenuated, and therefore more sensitive indicator of nucleic  
10 acid damage caused by ultraviolet irradiation to normal human melanocytes. Pre-treatment of such melanocytes with beta-carotene showed a statistically significant protective effect, particularly after one hour following UVB irradiation, at 500 mJ/cm<sup>2</sup>. The protective effect was statistically significant at a p value of less than 0.05 (using the Student's t-test). Accordingly, it appears that the NF Kappa B complex is the most appropriate model  
15 currently known for studying the conversion of melanocytes to melanomas, and the suppression of that process by beta-carotene.

## THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A carotenoid agent for inhibiting the conversion of epithelial cells to tumours, the agent including an effective amount of a water insoluble carotenoid component in a suitable non-toxic carrier medium.
- 5 2. A carotenoid agent as claimed in claim 1, wherein the epithelial cell is of mammalian origin.
3. A carotenoid agent as claimed in either of claims 1 or 2, wherein the epithelial cell is a skin cell.
- 10 4. A carotenoid agent as claimed in any one of the preceding claims, wherein the epithelial cell is a melanocyte, and the tumour is a melanoma.
5. A carotenoid agent as claimed in any one of the preceding claims, wherein the epithelial cell is of human origin.
6. A carotenoid agent as claimed in any of the preceding claims, wherein the water insoluble carotenoid component includes beta-carotene.
- 15 7. A carotenoid agent as claimed in any of the preceding claims, wherein the water insoluble carotenoid component includes from 2% to 50% by weight of beta-carotene.
8. A carotenoid agent as claimed in claim 7, wherein the water insoluble carotenoid component includes from 20% to 40% by weight of beta-carotene.
- 20 9. A carotenoid agent as claimed in either of claims 7 or 8, wherein the water insoluble carotenoid component includes 30% by weight of beta-carotene.
10. A carotenoid agent as claimed in any one of the preceding claims, wherein the carrier medium includes a suspending agent selected from the group comprising fatty acids, triglyceride lipids, non-saponifiable lipid preparations, soluble hydrocarbons, and combinations thereof.
- 25 11. A carotenoid agent as claimed in claim 10, wherein the triglyceride lipids are selected from the group comprising fats and/or oils derived from plant sources.
12. A carotenoid agent as claimed in claim 11, wherein the fats and/or oils are derived from seed oils.
- 30 13. A carotenoid agent as claimed in claim 12, wherein the seed oils include soya bean oil, cotton seed oil, sunflower oil and combinations thereof.
14. A carotenoid agent as claimed in claim 10, wherein the triglyceride lipids are selected from the group comprising fats and/or oils derived from animal sources.

15. A carotenoid agent as claimed in claim 14, wherein the fats and/or oils are derived from the group including meat and fish.
16. A carotenoid agent as claimed in claim 13, wherein the seed oil is soya bean oil.
17. A carotenoid agent as claimed in any of the preceding claims, wherein the water insoluble carotenoid component constitutes from 0.1% to 10% by weight of the agent.
18. A carotenoid agent as claimed in claim 17, wherein the water insoluble carotenoid component constitutes from 1% to 5% by weight of the agent.
19. A carotenoid agent as claimed in any of claims 16 to 18, wherein the water insoluble carotenoid component includes beta-carotene.
20. A carotenoid agent as claimed in any of the preceding claims, wherein the non-toxic carrier medium additionally includes an emulsifier.
21. A carotenoid agent as claimed in claim 20, wherein the emulsifier is selected from the group including Tweens, glycerol fatty acid esters and acetylated esters of fatty acids.
22. A carotenoid agent as claimed in claim 21, wherein the emulsifier is glycerol mono-oleate.
23. A carotenoid agent as claimed in any one of claims 20 to 22, wherein the non-toxic carrier medium also includes a water soluble dispersing agent.
24. A carotenoid agent as claimed in claim 23, wherein the water soluble dispersing agent is a sugar or a polyol.
25. A carotenoid agent as claimed in claim 24, wherein the water soluble dispersing agent is selected from the group including sorbitol and glycerol.
26. A method of inhibiting the conversion of epithelial cells into tumours, including the step of administering to the cells, an effective amount of a water insoluble carotenoid agent in a suitable non-toxic carrier medium.
27. A method of treating epithelial cells so as to inhibit their conversion into tumours, including the step of administering to the cells, an effective amount of a water insoluble carotenoid agent in a suitable non-toxic carrier medium.
28. A method as claimed in either of claims 26 or 27, wherein the epithelial cells are mammalian cells.
29. A method as claimed in any one of claims 26 to 28, wherein the epithelial cells are skin cells.



30. A method as claimed in any one of claims 26 to 29, wherein the epithelial cells are melanocytes.
31. A method as claimed in claim 30, wherein the melanocytes are human melanocytes.
- 5 32. A method as claimed in any one of claims 26 to 31, wherein the water insoluble carotenoid component includes beta-carotene.
33. A method as claimed in any one of claims 26 to 32, wherein the agent additionally includes an emulsifier.
34. A method as claimed in claim 33, wherein the emulsifier is selected from the group including Tweens, glycerol fatty acid esters and acetylated esters of fatty acids.
- 10 35. A method as claimed in claim 34, wherein the emulsifier is glycerol mono-oleate.
36. A method as claimed in any one of claims 26 to 35, wherein the non-toxic carrier medium also includes a water soluble dispersing agent.
37. A method as claimed in claim 36, wherein the water soluble dispersing agent is a sugar or a polyol.
- 15 38. A method as claimed in claim 37, wherein the water soluble dispersing agent is selected from the group including sorbitol and glycerol.
39. A method as claimed in any one of claims 26 to 38, wherein the water insoluble carotenoid component includes from 2% to 50% by weight of beta-carotene.
- 20 40. A method as claimed in claim 39, wherein the water insoluble carotenoid component includes from 20% to 40% by weight of beta-carotene.
41. A method as claimed in either of claims 39 or 40, wherein the water insoluble carotenoid component includes 30% by weight of beta-carotene.
- 25 42. A method as claimed in any one of claims 26 to 41, wherein the carrier medium includes a suspending agent selected from the group comprising fatty acids, triglyceride lipids, non-saponifiable lipid preparations, soluble hydrocarbons, and combinations thereof.
43. A method as claimed in claim 42, wherein the triglyceride lipids are selected from the group comprising fats and/or oils derived from plant sources.
- 30 44. A method as claimed in claim 43, wherein the fats and/or oils are derived from seed oils.
45. A method as claimed in claim 44, wherein the seed oils include soya bean oil, cotton seed oil, sunflower oil and combinations thereof.

46. A method as claimed in claim 42, wherein the triglyceride lipids are selected from the group comprising fats and/or oils derived from animal sources.
47. A method as claimed in claim 45, wherein the fats and/or oils are derived from the group including meat and fish.
- 5 48. A method as claimed in claim 44, wherein the seed oil is soya bean oil.
49. A method as claimed in any one of the preceding claims, wherein the water insoluble carotenoid component constitutes from 0.1% to 10% by weight of the agent.
- 10 50. A method as claimed in claim 49, wherein the water insoluble carotenoid component constitutes from 1% to 5% by weight of the agent.
51. A method as claimed in either of claims 50 or 51, wherein the carotenoid component includes beta-carotene.
52. A carotenoid agent as claimed in any one of claims 1 to 25, wherein the effective amount of the carotenoid agent contacting the cells is from 0.1 to 10.0 micrograms/ml.
- 15 53. A carotenoid agent as claimed in claim 52, wherein the effective amount of the agent is from 0.3 to 3.0 micrograms/ml.
54. A method as claimed in any one of claims 26 to 51, wherein the effective amount of the carotenoid agent contacting the cells is from 0.1 to 10.0 micrograms/ml.
- 20 55. A method as claimed in claim 54, wherein the effective amount of the agent is from 0.3 to 3.0 micrograms/ml.

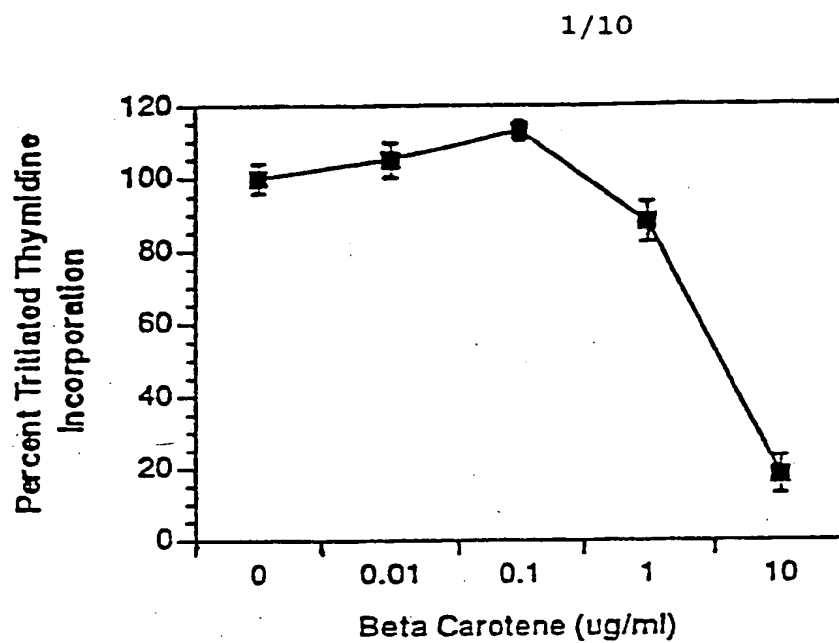
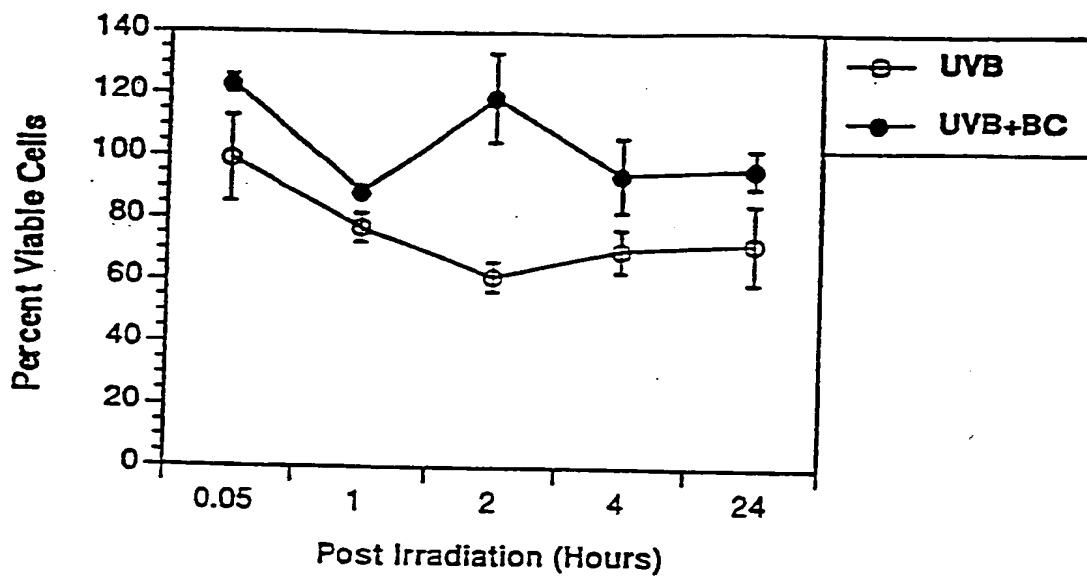


FIGURE 1

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FIGURE 2

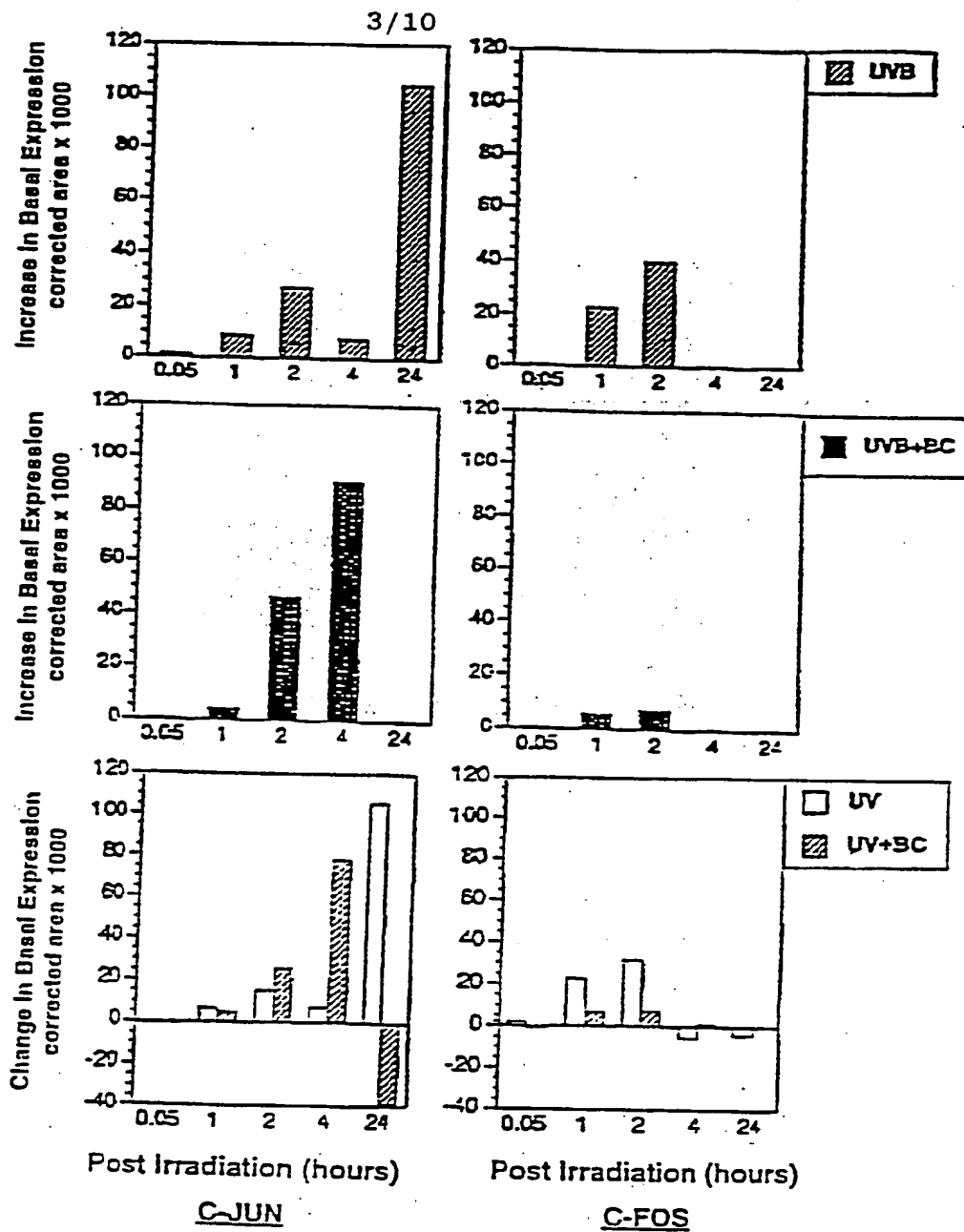
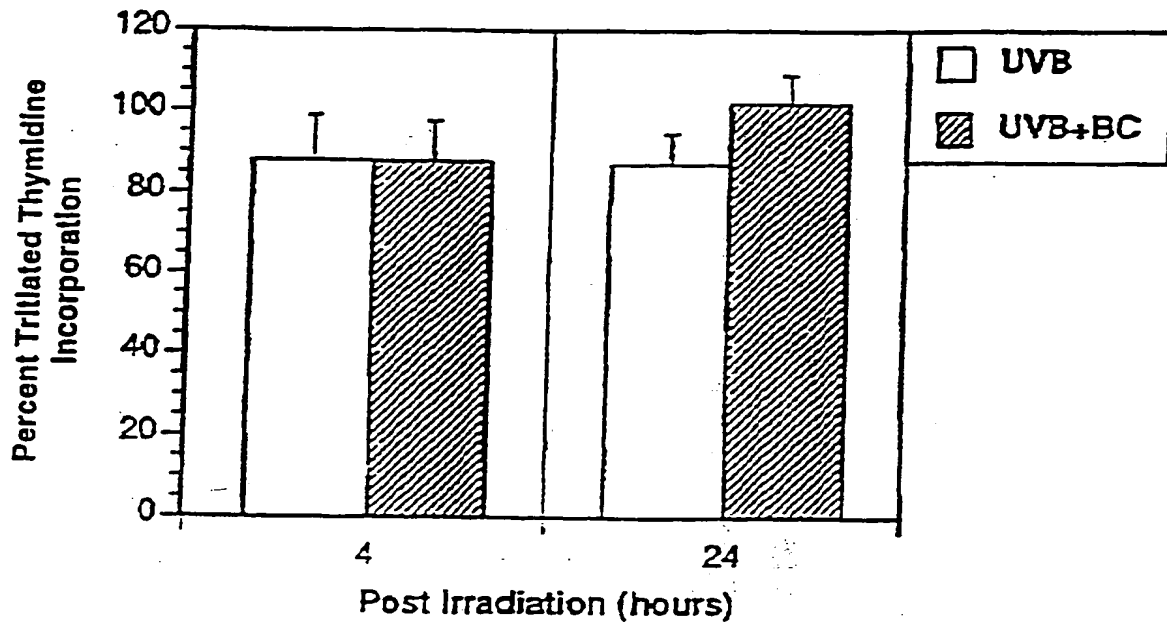


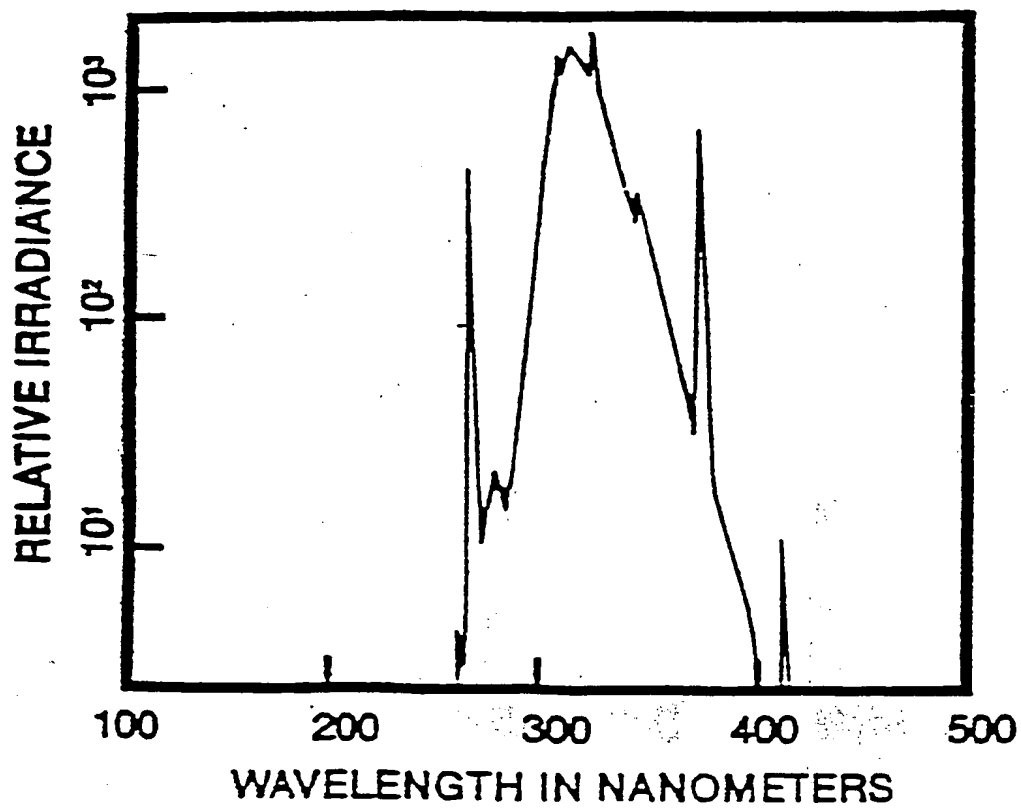
FIGURE 3

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FIGURE 4

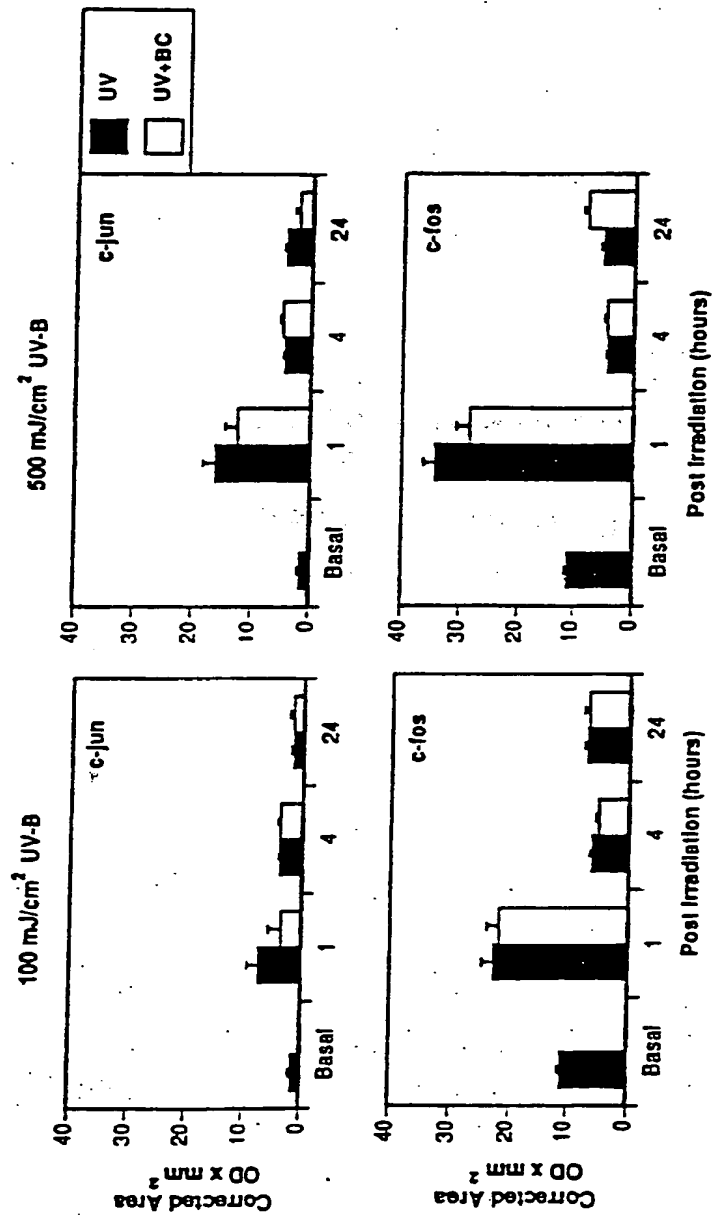
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302nm

FIGURE 5

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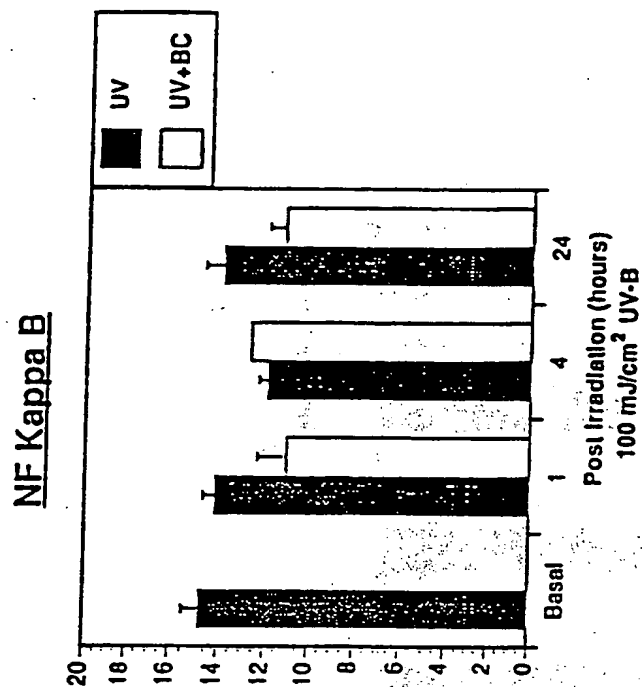
FIGURE 6





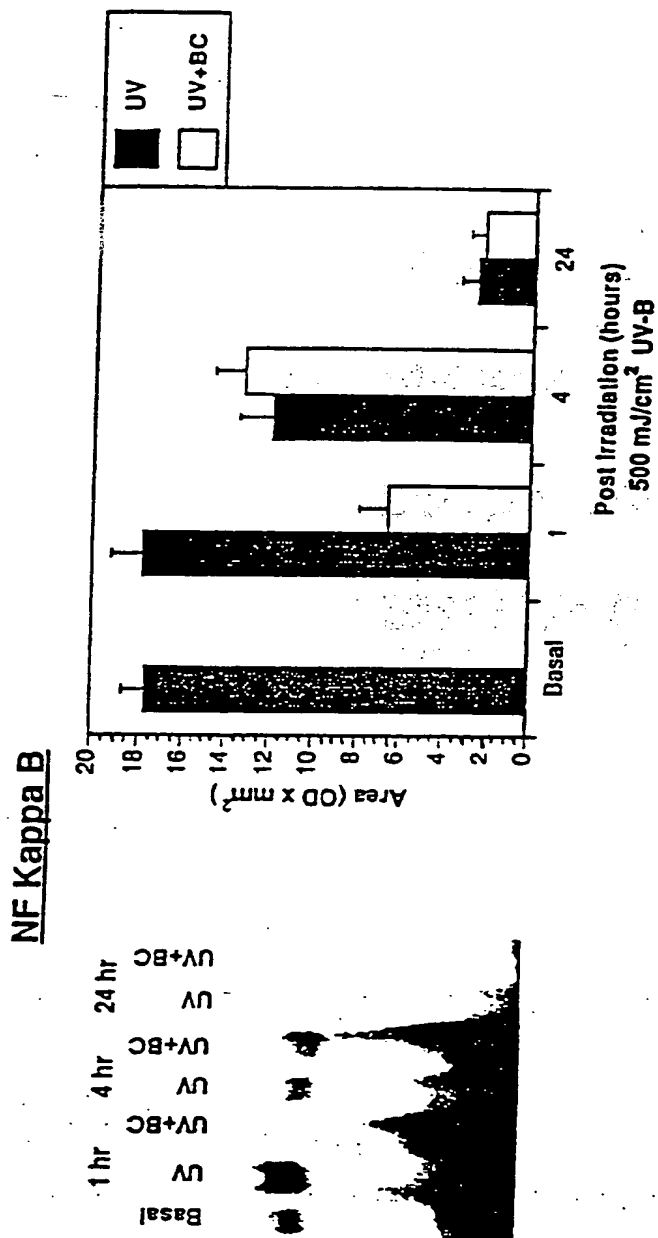
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FIGURE 7



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FIGURE 8



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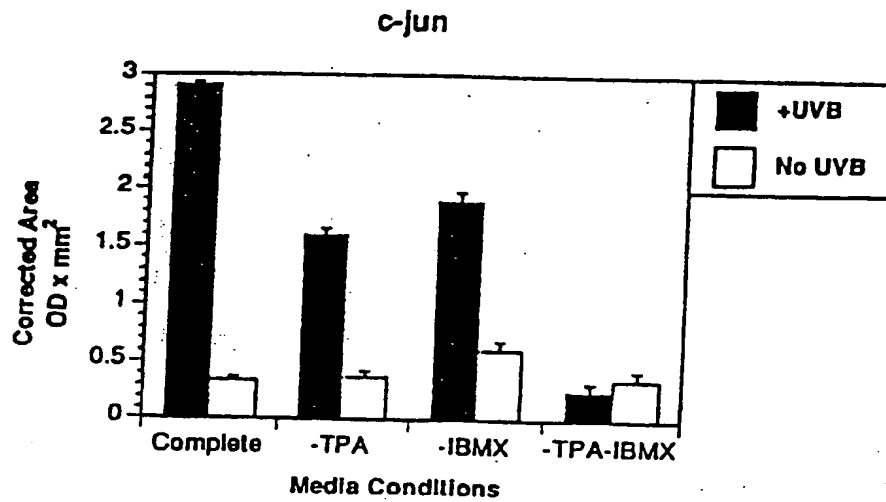
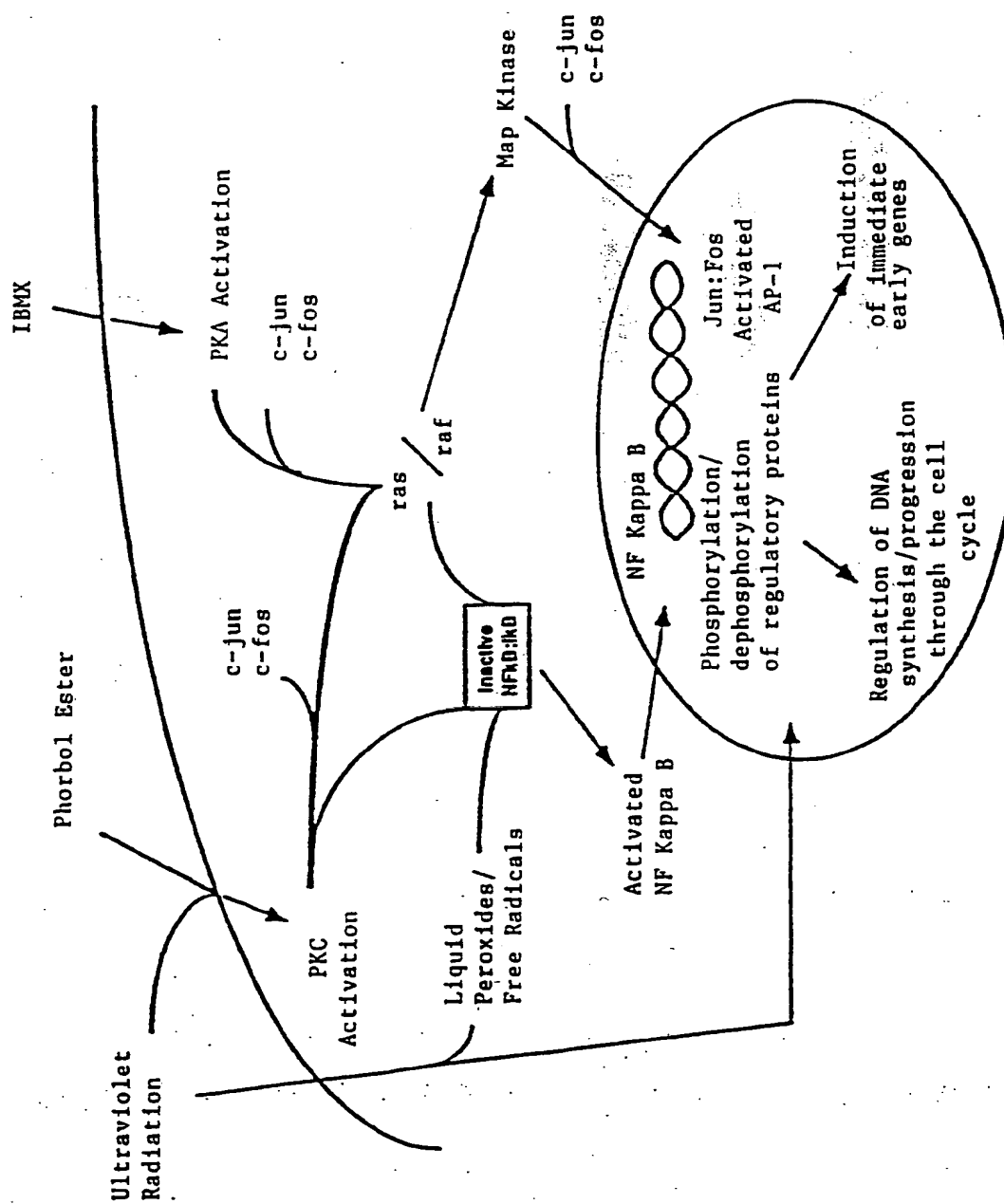


FIGURE 9

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FIGURE 10



# INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU 95/00199

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> Int. Cl. <sup>6</sup> A61K 31/015 31/07  According to International Patent Classification (IPC) or to both national classification and IPC				
<b>B. FIELDS SEARCHED</b>  Minimum documentation searched (classification system followed by classification symbols) A61K 31/015 31/07  Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched AU: IPC as above  Electronic data base consulted during the international search (name of data base, and where practicable, search terms used) DERWENT WPAT: cancer: neoplas: carcin: caroten: tumour: melanom: CHEM ABS CASM: cancer: neoplas: carcin: caroten: tumour: melanom:				
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>				
<b>Category*</b>	<b>Citation of document, with indication, where appropriate, of the relevant passages</b>	<b>Relevant to Claim No.</b>		
P, X	WO 94/21231 A1 (BETATENE LIMITED), 29 September 1994 Page 7 and claims	1-55		
P, X	WO 94/21232 A1 (BETATENE LIMITED), 29 September 1994 Page 7 and claims 10-19	1-25 & 52-53		
<div style="display: flex; justify-content: space-between;"> <div> <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C.         </div> <div> <input checked="" type="checkbox"/> See patent family annex.         </div> </div>				
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; vertical-align: top;"> <p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </td> <td style="width: 50%; vertical-align: top;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p> </td> </tr> </table>			<p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p>
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Date of the actual completion of the international search 24 May 1995		Date of mailing of the international search report 19 JUNE 1995 (19.06.95)		
Name and mailing address of the ISA/AU  AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA  Facsimile No. 06 2853929		Authorized officer  GILLIAN JENKINS  Telephone No. (06) 2832252		

## INTERNATIONAL SEARCH REPORT

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
P, X	WO 94/08631 A1 (ARIZONA BOARD OF REGENTS), 28 April 1994 Pages 3-5 and claims	1-10, 17-19, 20, 27, 33 & 49-55
P, Y		11-16, 21-26, 28-32 & 34-48
X	JP 5-124958 A (YUKIO DATE), 21 May 1993 The abstract	1-13, 17-19, 26-28, 39-45 & 48-55
Y		14-16, 20-25, 29-38, 46-47
X	EP 0467795 A2 (PATRINOVE) Example 1 and claims	1-13, 16-21, 26-34, 39-45 & 48-55
Y		14-15, 22-25, 35-38 & 46-47
X	JP 4-120019 A (TAKAO MATSUNO), 21 April 1992 The abstract	1-13, 26-31 & 42
Y		14-25, 32-41 & 43-55
X	WO 89/12626 A1 (HERMAN), 28 December 1989 The abstract and claims 3, 10 & 17	1-13, 17-45 & 49-55
Y		14-16 & 46-48
X	US 4680314 A1 (NONOMURA), 14 July 1987 Column 2, line 55 - column 3 line 46 and claims	1-22, 26-27, 32-35, 39-45 & 48-55
Y		23-25, 28-31, 36-38 & 46-47
X	GB 1509587 A (TSUYOSHI SHIIO et al), 4 May 1978 Page 1, lines 6-12, Example 1, claims 1 & 9	1-9, 26, 28-32, 39-41 & 49-55
Y		11-25, 33-38 & 42-48

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